

c-JUN Expression and Apoptotic Cell Death in Kainate-Induced Temporal Lobe Epilepsy

Following kainate (KA)-induced epilepsy, rat hippocampal neurons strongly express immediate early gene (IEG) products, i.e., c-FOS and c-JUN, and neural stress protein, HSP72. Prolonged expression of c-JUN and c-FOS 48 hr after cerebral ischemia has been underwent delayed neuronal death. However, it is not yet clear whether IEGs actually assume the essential roles in the cell death process or simply as a by-product due to external stimuli because of the prolonged expression of c-FOS, more than one week, on intact CA2 neurons of the hippocampus in a KA-induced epilepsy model. This study investigated the relationships between prolonged expression of c-JUN and hippocampal neuronal apoptosis in a KA-induced epilepsy model. Epileptic seizure was induced in rats by a single microinjection of KA (1 $\mu\text{g}/\mu\text{L}$) into the left amygdala. Characteristic seizures and hippocampal neuronal injury were developed. The expression of c-JUN was evaluated by immunohistochemistry, and neuronal apoptosis by in situ end labeling. The seizures were associated with c-JUN expression in the hippocampal neurons, of which the level showed a positive correlation with that of apoptosis. Losses of hippocampal neurons, especially in the CA3 region, were partly caused by apoptotic cell death via a c-JUN-mediated signaling pathway. This is thought to be an important component in the pathogenesis of hippocampal neuronal injury via KA-induced epilepsy.

Key Words : Apoptosis; Genes, Immediate-Early; Epilepsy; Hippocampus; Kainic Acid

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INTRODUCTION

Kainate (kainic acid, KA) is an excitotoxic analogue of glutamate extracted from the seaweed *Digenea simplex* (1). A microinjection of KA into the unilateral amygdala or hippocampus results in early limbic seizures and late secondary generalized seizures (2, 3). KA-induced late epileptic seizures in the rat closely resemble the behavioral and neuropathological alterations of temporal lobe epilepsy in humans (4). Therefore, the KA-induced epilepsy model is frequently used to study the pathophysiology of localization-related, partial or focal, epilepsies (5-7).

Chronic temporal lobe epilepsy in humans and prolonged, repeated KA-induced seizures both produce hippocampal sclerosis characterized by the loss of neurons associated with gliosis in the hippocampus (4, 8-10). Seizure-induced neuronal death most likely involves an excitotoxic mechanism activated by the N-methyl-D-aspartate or kainate/AMPA subtype of glutamate receptors, leading to a rise in intracellular calcium with subsequent calcium-activated intracellular signaling pathways (10-12). Further downstream in these

intracellular signal transduction schemes, alteration of gene expression may contribute to a molecular mechanism causing hippocampal neuronal damage (13, 14).

A subset of cellular immediate early genes (IEGs), *c-fos* and *c-jun*, encode transcription factors which interact with the transcriptional regulatory element, activation protein-1 (AP-1) (15). Early upregulation of *c-fos* and *c-jun* transcription, from 15 to 45 min, along with the expression of their gene products, from 1 hr up to 3 days, have been demonstrated in hippocampal neurons in experimental seizures (16-18) or cerebral ischemia (19). However, the specific roles of IEGs have not yet been elucidated. It is not clear if IEGs serve essential roles in the cell death process or simply appear nonspecifically in response to external stimuli (20). There is some evidence that prolonged expression of IEGs in selected vulnerable cells suggests neuronal cell death (21, 22). Through the KA-induced experimental epilepsy model, this study investigates the relationship between prolonged c-JUN expression and apoptotic cell death in hippocampal neurons.

MATERIALS AND METHODS

Stereotactic operation and injection of kainate

Adult male Wistar rats, 250-300 g, were anesthetized with pentobarbital sodium (Nembutal, Abbott, Osaka, Japan, 50 mg/kg i.p.), fixed on a stereotactic frame (David-Kopf, U.S.A.), and stereotactic operations were performed. A stainless steel cannula with internal stylet for KA (Nacal Tesque, Kyoto, Japan) microinjection, 0.03 mm in internal diameter, was implanted in the left amygdala using sterile techniques. Coordinates for the implantation target were: AP +5 mm, ML +5 mm and DV +2 mm with respect to the interaural zero point (23). The cannula was fixed with dental cement. The experimental animals (n=45) were left free for 7 days to recover from the operation.

KA was prepared immediately before each injection. KA crystals were dissolved in 0.2 M phosphate buffer solution (pH 7.4) at a concentration of 1 mg/mL and sterilized through a 0.45 μ m microfilter. The injection was delivered while the animals were awake and resting under aseptic conditions. Removing the inner guide wire from the cannula, an injection needle was inserted. Following the injection of KA (1 μ g/ μ L) into the left amygdala, successful administration was determined by the induction of clinical seizures. During the first hour after KA injection, animals exhibited "staring spells" followed by repetitive head nodding and "wet dog shakes". During the next 2 hr, progressive motor seizures developed, including masticatory and facial movements, tremors of the forepaws, rearing and loss of postural control. Finally, animals suffered from limbic status epilepticus with continuous convulsions, lasting 1 to 2 days. The seizures disappeared spontaneously 3 days after KA injection, and motor seizures developed again at about 4 weeks after the injection. During the initial postictal period, animals demonstrated reduced motor activity, but were otherwise normal. Thirty rats showing characteristic clinical features after KA injection were selected. Five rats were sacrificed in deep anesthesia at 3 days, 1, 2, 4, 8, and 16 weeks after the injection. The whole brain was taken immediately and fixed in 10% neutral buffered formalin. Routine paraffin blocks were made, and observed by H&E and Nissl stains. The right half of the brain was used as the control.

Immunohistochemistry for c-JUN

Paraffin sections (4 μ m thick) on probed-glass slides (Fisher Scientific, Pittsburg, PA, U.S.A.) were immunostained with anti-mouse monoclonal antibody for c-JUN using the avidin-biotin peroxidase complex (ABC) method (24). Paraffin was dissolved from the tissue slides using xylene. The endogenous peroxidase activity was suppressed by incubation in 0.3% hydrogen peroxide with 10% methanol. The tissue slides were rehydrated with graded alcohol, treated

with 10% normal goat serum for 30 min, then incubated with a primary antibody overnight at a temperature of 4°C. They were incubated with biotinylated anti-mouse IgG for 30 min at room temperature. The tissue slides were then treated with 1% avidin-biotinylated horseradish peroxidase in 10% normal goat serum for 1 hr at room temperature, and then developed in a mixture of 0.4 mg/mL 3,3-diaminobenzidine (DAB, Sigma) and 0.1% hydrogen peroxide solution for 5 min. All immunostained tissues were counterstained with hematoxylin solution for 5 min. After dehydration, the tissue was sealed with a universal mount (Research Genetics, Huntsville, AL, U.S.A.) and examined under a light microscope. About 50-250 cells in each region of the hippocampus were counted.

In Situ End Labeling (ISEL) for apoptosis

The extent of apoptosis in the hippocampal neurons was determined by the ISEL method (25) using the ApopTag In Situ Detection Kit (Oncor, Gaithersburg, MD, U.S.A.). This method relies upon the capacity of exogenous enzymes, such as DNA polymerase or terminal deoxynucleotidyl-transferase (TdT), to incorporate labeled nucleotides in the 3'-hydroxyl terminal of DNA breaks produced by endonuclease.

Sections (4 μ m thick) from formalin-fixed paraffin tissue blocks were mounted on glass slides previously labeled with probes, air-dried and incubated overnight at 45°C. After deparaffinization and rehydration, the sections were digested with proteinase K (120 μ g/mL) for 15 min at room temperature. The slides were then washed in distilled water and immersed in 2% hydrogen peroxide in distilled water for 5 min to quench the endogenous peroxidase activity. The sections were washed in phosphate buffered saline (PBS) and subsequently incubated with equilibration buffer for 10 min at room temperature. After blotting the sections, 40 μ L of a mixture containing TdT and the reaction buffer, containing dATP and digoxigenin-II-dUTP, was applied. The sections were placed in a humidified chamber at 37°C for 1 hr, and then washed in stop/wash buffer for 10 min at room temperature and subsequently in PBS. The sections were then incubated with anti-digoxigenin-peroxidase for 30 min at room temperature and were washed in PBS. Finally, the slides were developed by immersion in a mixture of 0.04% DAB and 0.1% hydrogen peroxide solution for 5 min. Sections were counterstained with Mayer hematoxylin and mounted with a universal mount (Research Genetics). Sections from lymph nodes with active follicles were used as positive controls, and sections containing no TdT were used as negative controls. Approximately 50-250 cells in each region of the hippocampus were randomly counted.

Statistical analysis

To compare the means of c-JUN and ISEL between CA3

and other area, ANOVA with multiple comparison was performed. Pearson's correlation analysis was applied to evaluate the relation between c-JUN and ISEL.

RESULTS

Histopathologic changes

The control side of the (right) hippocampus in the coronal section demonstrated well-defined neuronal arrangements consisting of CA1, CA2, CA3, CA4 and the dentate gyrus (DG). The KA injection side of the (left) hippocampus also appeared relatively intact for about 2 weeks. Pathologic changes of the hippocampus in routine H&E-stained tissue sections were evident 4 weeks after KA injection into the amygdala with swelling of the neuronal cytoplasm and axial dendrites and nuclear pyknosis in CA3. Few pyknotic neu-

rons were found in the DG. Neurons in CA1 and CA4 were minimally involved, and CA2 neurons were relatively intact. A decrease in the number of neurons in CA3 was noted 8 weeks after KA injection (Fig. 1), and significant neuronal loss was obvious at 16 weeks.

c-JUN expression

Immunoreactivity for c-JUN was restricted to neuronal cells. On the control side of the hippocampus, only a few (< 1% of neurons) c-JUN immunoreactive neurons were scattered throughout the DG, CA1 and CA4. There was no significant difference between the experimental periods.

Following KA injection into the amygdala, an increased number of c-JUN-positive neurons were observed in CA3, DG, CA1 and CA4 (Fig. 2-5). Immunoreactivity showed peak levels in CA3 comparing with other area of the hippocampus after KA injection, and constituted 8.6% of the

Table 1. Mean percentages of c-JUN positive cells and apoptotic cells (ISEL) in the hippocampus after kainic acid (1 $\mu\text{g}/\mu\text{L}$) injection into the ipsilateral amygdala

Time	CA1		CA3		CA4		DG	
	c-JUN	ISEL	c-JUN	ISEL	c-JUN	ISEL	c-JUN	ISEL
3 D	3.66 \pm 0.30*	3.10 \pm 0.57*	8.58 \pm 0.94	4.76 \pm 0.81	4.52 \pm 0.80*	1.86 \pm 0.64*	6.00 \pm 0.35	2.92 \pm 0.74*
1 W	2.52 \pm 0.55*	1.78 \pm 0.58*	5.48 \pm 0.89	3.34 \pm 0.71	3.24 \pm 0.50	2.16 \pm 0.58	2.70 \pm 0.85*	1.06 \pm 0.53*
2 W	1.74 \pm 0.53*	1.18 \pm 0.58*	4.70 \pm 0.97	4.18 \pm 0.85	1.56 \pm 0.68*	1.06 \pm 0.59*	3.18 \pm 0.58	2.28 \pm 0.48
4 W	4.64 \pm 0.89	2.02 \pm 0.65*	7.08 \pm 0.93	5.30 \pm 0.95	2.38 \pm 0.70*	1.80 \pm 0.56*	7.60 \pm 0.99	2.54 \pm 0.63*
8 W	3.74 \pm 0.86*	1.72 \pm 0.56*	7.72 \pm 0.53	3.92 \pm 0.80	2.92 \pm 0.51*	1.70 \pm 0.68*	7.98 \pm 0.85	2.74 \pm 0.49
16 W	3.04 \pm 0.82*	1.92 \pm 0.58*	6.82 \pm 1.04	3.22 \pm 0.63	3.54 \pm 0.83*	2.18 \pm 0.68	5.50 \pm 0.75	2.78 \pm 0.73

c-JUN, c-JUN positive cells (%) detected by immunohistochemical stain. ISEL, apoptotic cells (%) by *in situ* end labeling method. D, days; W, week; DG, dentate gyrus. *, $p < 0.05$ between CA3 and other location of the hippocampus in multiple comparison test

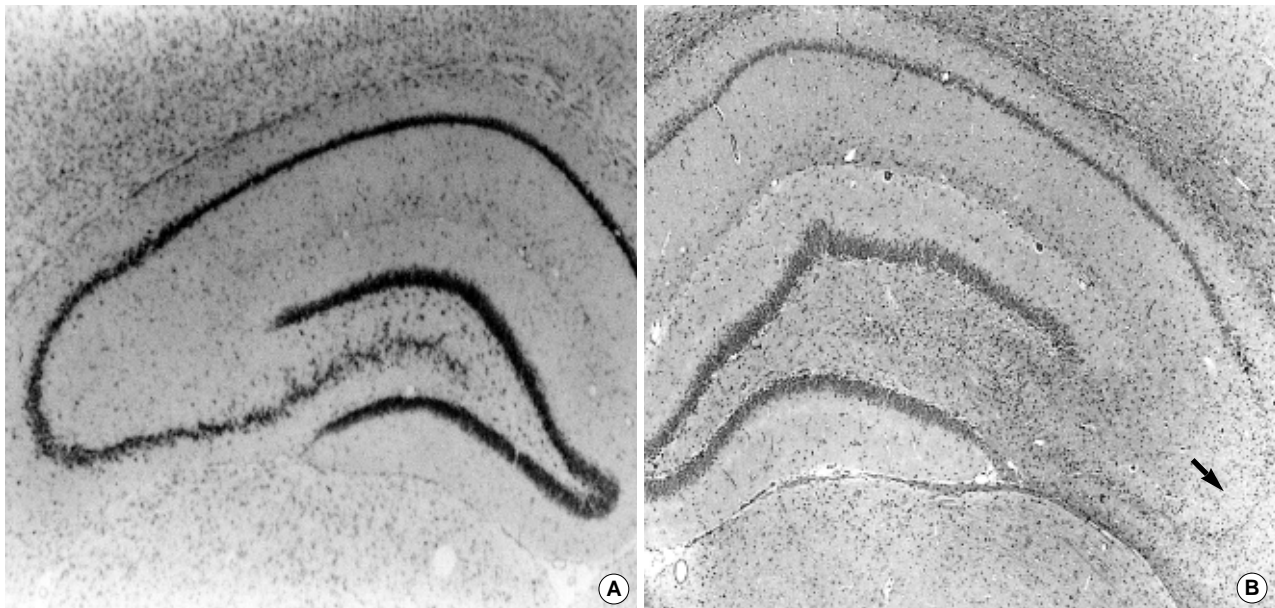


Fig. 1. Coronal section of the hippocampus, 8 weeks after KA injection, reveals relatively intact neurons on right side (A, Nissl stain, $\times 40$) and significant loss of CA3 neurons (arrow) on left side (B, H&E, $\times 40$).

neurons at 3 days (Table 1). Moderately increased immunoreactivity appeared 4-16 weeks after KA injection, with a notable 7-8% positivity in CA3.

Apoptotic cells

Apoptotic cells were scarce on the control side of the hippocampus, constituting 0-0.5% of the observed neurons.

No significant difference was noted between the different experimental periods.

Following KA injection into the amygdala, the number of ISEL-positive neurons increased in CA3, DG, CA1 and CA4 (Fig. 2, 4, 5), but not in CA2 (Fig. 3, less than 0.5%). Much more apoptotic cells were present in CA3 than in other regions of the hippocampus (Table 1). The highest number of apoptotic cells was noted in CA3 at 4 weeks after KA-

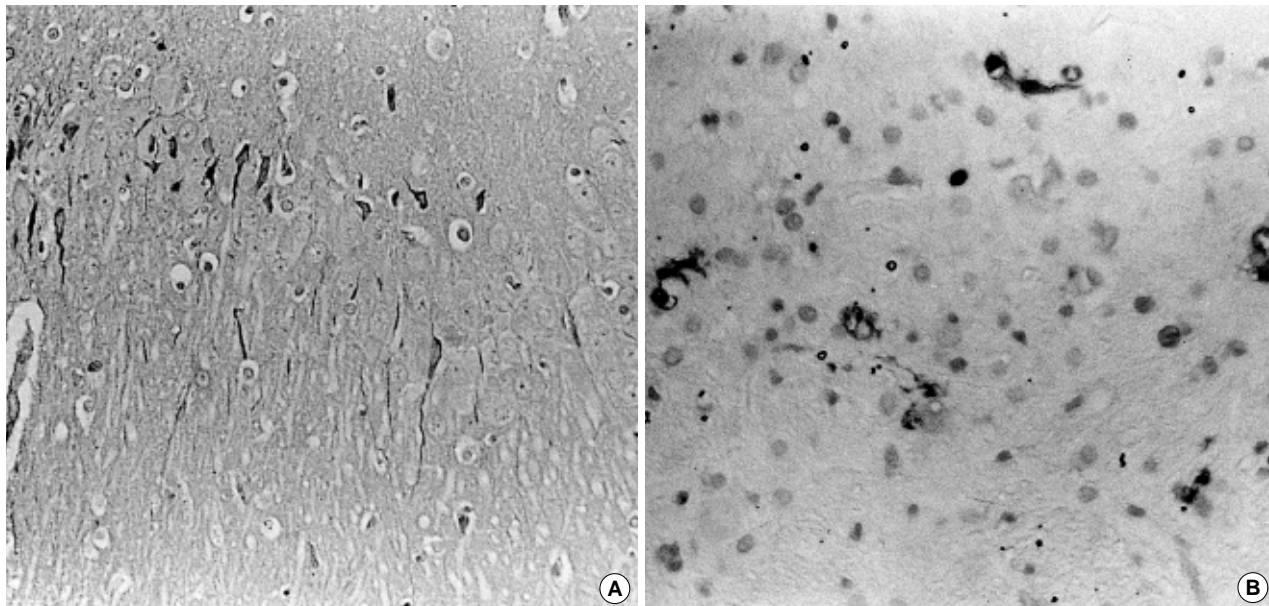


Fig. 2. Pyramidal neurons in CA1, 4 weeks after KA injection, reveal c-JUN positive cells (A, c-JUN immunohistochemistry, $\times 80$) and apoptotic cells (B, ISEL, $\times 160$).

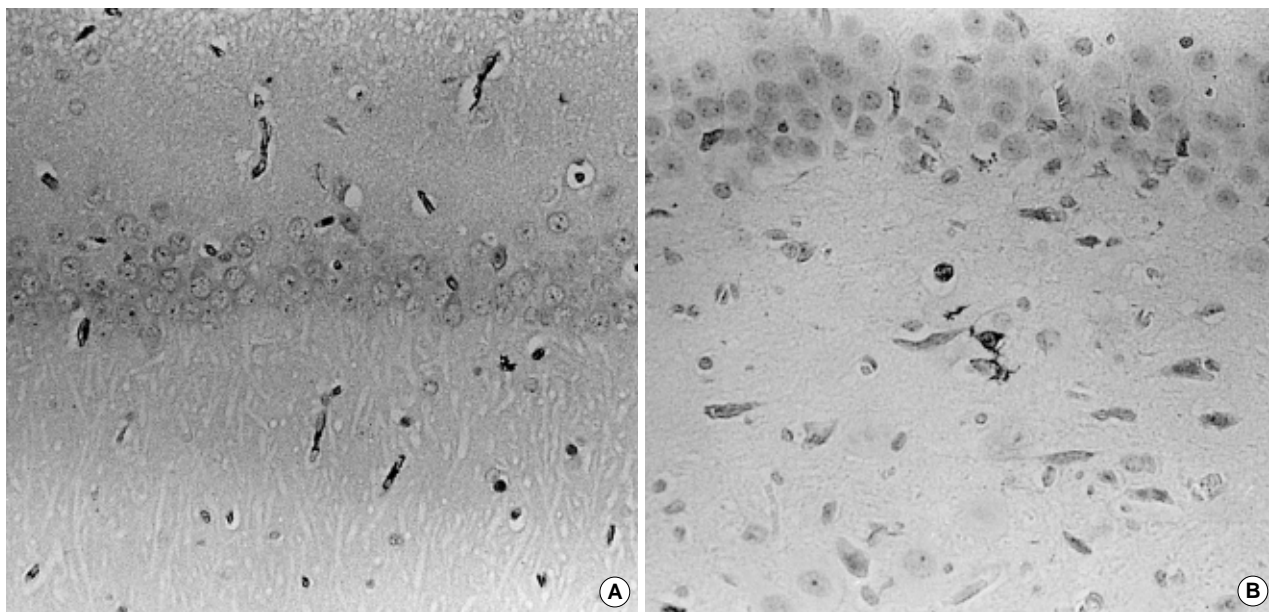


Fig. 3. Pyramidal neurons in CA2, 8 weeks after KA injection, disclose only a few c-JUN positive cells (A, c-JUN immunohistochemistry, $\times 20$) and absence of apoptotic cells (B, ISEL, $\times 180$).

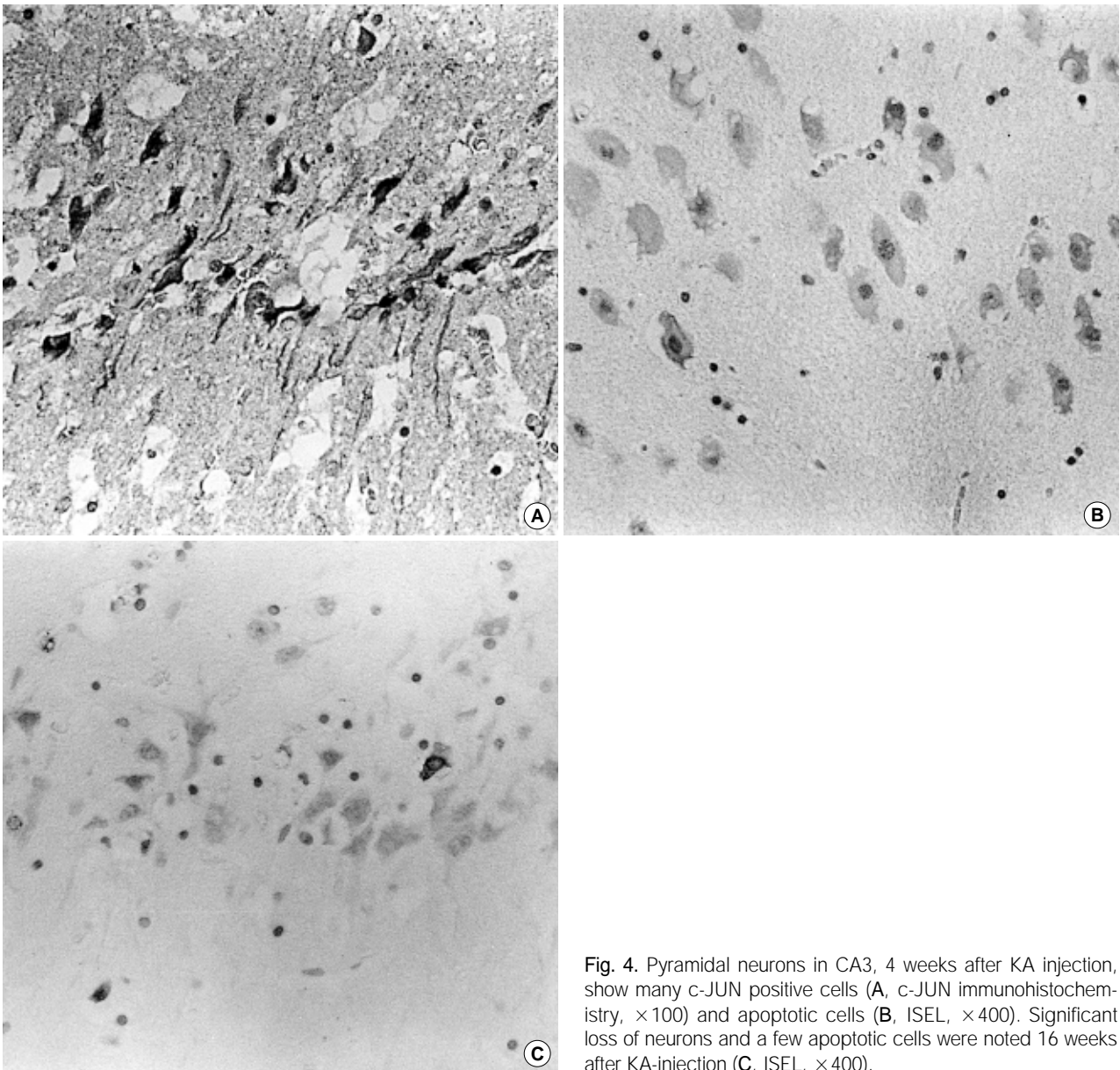


Fig. 4. Pyramidal neurons in CA3, 4 weeks after KA injection, show many c-JUN positive cells (A, c-JUN immunohistochemistry, $\times 100$) and apoptotic cells (B, ISEL, $\times 400$). Significant loss of neurons and a few apoptotic cells were noted 16 weeks after KA-injection (C, ISEL, $\times 400$).

injection (5.3%).

Levels of c-JUN expression and ISEL-positive apoptotic cells in the hippocampal neurons following KA injection into the ipsilateral amygdala are summarized in Table 1.

Statistical correlation between c-JUN and ISEL

In the quantitative expression of c-JUN and ISEL in the experiment, there was an interaction effect between the time interval and location of the hippocampus. It meant significant differences in c-JUN and ISEL between CA3 and other areas depend on time interval of the study (Table 1, Fig. 6 and 7). The correlation between c-JUN and ISEL was statistically significant in CA1, 3, 4 and DG (Fig. 8,

$r=0.64$, $p<0.01$).

DISCUSSION

A single microinjection of KA into the unilateral amygdala in rats produced characteristic seizures and hippocampal neuronal injury. The characteristic course of the seizures consisted of transient limbic seizures for about 2 to 3 days, transient seizure-free periods for about 3 to 4 weeks, and finally the spontaneous appearance of generalized hippocampal neuronal loss especially in CA3 region. The symptomatology and histopathologic changes correlated well with those in other experiments (3, 26, 27). The seizures were

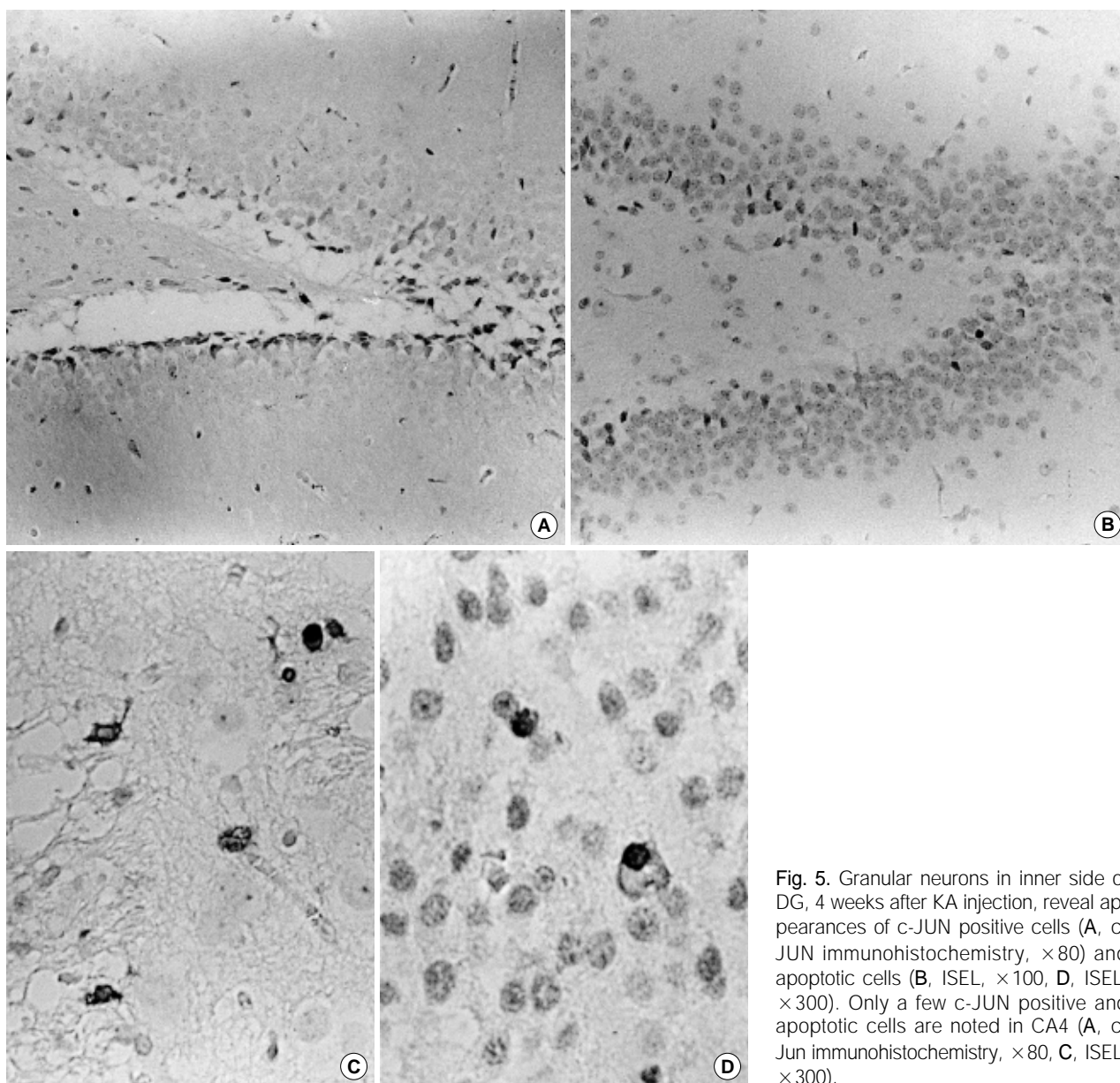


Fig. 5. Granular neurons in inner side of DG, 4 weeks after KA injection, reveal appearances of c-JUN positive cells (A, c-JUN immunohistochemistry, $\times 80$) and apoptotic cells (B, ISEL, $\times 100$, D, ISEL, $\times 300$). Only a few c-JUN positive and apoptotic cells are noted in CA4 (A, c-JUN immunohistochemistry, $\times 80$, C, ISEL, $\times 300$).

associated with c-JUN expression in the hippocampal neurons. There were positive correlations between the quantitative expression of c-JUN and apoptosis. Loss of hippocampal neurons especially in CA3 was caused in part by apoptotic cell death.

Remote hippocampal lesions following the intra-amygdaloid injection of KA might be caused by powerful excitatory action on CA3 pyramidal neurons via unidirectional amplifying neural circuits: amygdala \rightarrow entorhinal cortex \rightarrow granule cells and mossy fibers \rightarrow CA3 neurons (4). Other factors of neuronal cell loss include the uncoupling of local glucose utilization and blood flow, subsequent hypoxia, lactic acidosis, capillary endothelial damage and edema (1, 28, 29).

A wide range of pathologic insults to the brain, including trauma, ischemia and seizures induce the IEGs, *c-fos*, *c-jun* and *jun B* (30-32). Due to the short half-life of IEG encoded mRNAs, researchers prefer immunocytochemical investigations on IEG proteins to in situ hybridization studies (33, 34). According to epilepsy studies on rats through systemic administration of excitotoxic agents, three major categories of IEG-encoded transcription factors can be defined by the timing of their induction in the hippocampus (21, 34, 35). First c-FOS and KROX-24 show a concurrent rapid rise with peak levels at 2 hr of postictal recovery, and decline to control levels within 8 hr after seizure termination. Second, c-JUN and JUN B demonstrate a more gradual increase with peak intensities at 4 hr and return to baseline levels

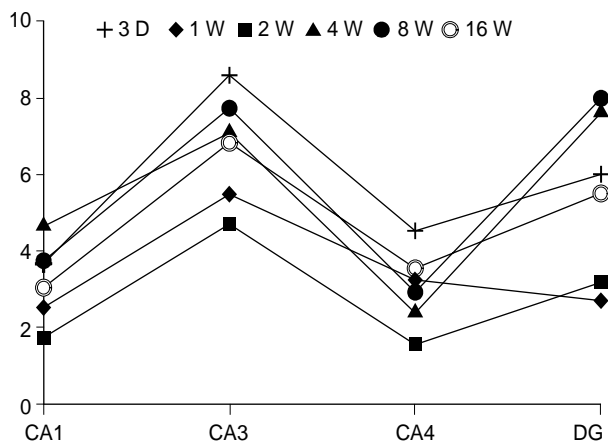


Fig. 6. Distribution of c-JUN by time and location.

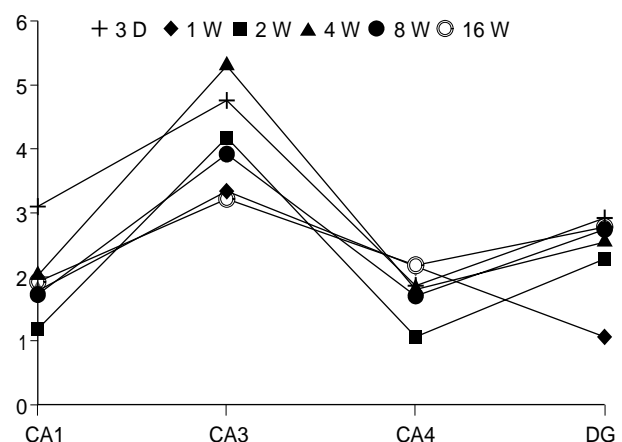


Fig. 7. Distribution of ISEL by time and location.

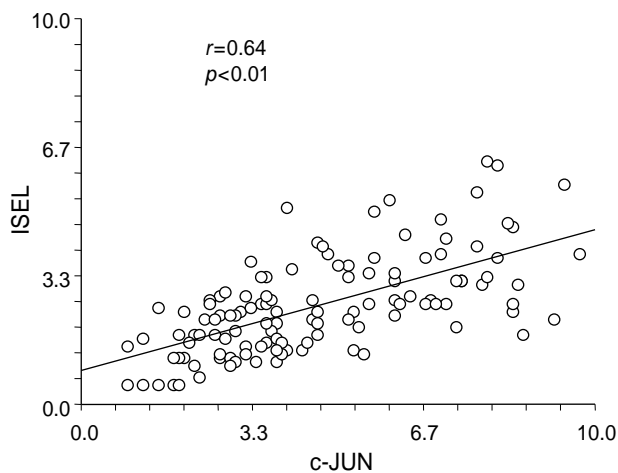


Fig. 8. Correlation between c-JUN and ISEL.

after 24 hr of postictal recovery. Third, FOS B, JUN D and KROX 20 exhibit a delayed induction and prolonged persistence up to 24 hr after seizure termination. All IEG products, with the exception of KROX-20, demonstrate the same sequence of induction in different hippocampal subfields, following the order of DG, CA1 and CA3.

The most prevalent expression of c-JUN was noted in CA3 of the hippocampus. Peak levels were observed at 3 days and 4-8 weeks after KA injections, when transient limbic seizures disappeared and spontaneous generalized seizures began and continued, respectively. Expression of c-JUN correlated with seizure activity. The more delayed expression of c-JUN in this study relative to other studies could be explained by the route of KA injection or a small amount of the injected kainate.

Apoptosis is a distinct form of controlled cell degeneration, which is different from necrosis (36, 37). Gene regulation of apoptosis varies among different tissues, particularly regarding the signals that trigger cell death. However, there is a common effector phase. In the nervous system, genes

have been identified which either i) promote apoptosis: *Bax*, *Bcl-xs*, *c-fos*, *c-jun*, p75NGFR and ICE-like proteases, or ii) block apoptosis: *Bcl-2* and *Bcl-xL*. In addition, the availability of trophic factors and the expression of Trk membrane receptors allow for the fine adjustment of viable cells in each neuronal population (38). The results of this study revealed an increased number of apoptotic cells in CA3 (3.2-5.3%) relative to the DG (1.1-2.9%) and CA1 (1.2-3.1%). Apoptosis could account for the significant loss of neurons in CA3 and the relatively intact neurons in CA2 in the hippocampus. A previous study demonstrated frequent c-FOS immunoreactivity in CA3 as well as CA2 neurons in rats (18), however, c-JUN immunoreactivity was not correlated with c-FOS in CA2 neurons. These observations indicate that hippocampal neuronal loss, most severe in CA3, may be caused by apoptotic cell death via a c-JUN-mediated signaling pathway. This is an important component in the pathogenesis of hippocampal neuronal injury in KA-induced epilepsy.

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